



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/53, C07K 16/00, A61K 39/395</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/02991</b> <b>(43) International Publication Date:</b> 21 January 1999 (21.01.99)
<b>(21) International Application Number:</b> PCT/US98/14216 <b>(22) International Filing Date:</b> 9 July 1998 (09.07.98) <b>(30) Priority Data:</b> 60/052,132      10 July 1997 (10.07.97)      US <b>(71) Applicant:</b> RESEARCH DEVELOPMENT FOUNDATION [US/US]; 402 North Division Street, Carson City, NV 89703 (US). <b>(72) Inventors:</b> SANDERS, Robert; 2005 Barton Hills Drive, Austin, TX 78726 (US). KLINE, Kimberly; 2505 McCal- lum, Austin, TX 78703 (US). <b>(74) Agent:</b> WEILER, James, F.; Suite 1560, 1 Riverway, Houston, TX 77056 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ANTIBODIES AND SCFV IMMUNOTOXINS SPECIFIC TO IMPORTED FIRE ANTS, AND THEIR APPLICATION <b>(57) Abstract</b> <p>The present invention is drawn to a safe, cost-effective, environmentally-friendly and ecologically-sound bioengineered product for managing imported fire ants, and a method of making this product. Immunological and genetic engineering techniques are used to generate monoclonal antibodies (mAbs) as well as viruses (phage) that display scfv heavy and/or light chain Ig fragments which exhibit high-avidity specific binding to cells of the microvilli of the midgut of imported fire ant queens. The specific monoclonal antibodies and phage displayed antibody Fab fragments are conjugated to a toxin for targeted delivery and destruction of imported fire ant queens, but not native species, thereby restoring the natural ecosystem.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

5

ANTIBODIES AND SCFV IMMUNOTOXINS SPECIFIC TO IMPORTED FIRE ANTS, AND THEIR APPLICATION

10

15

20

## BACKGROUND OF THE INVENTION

### 25 Field of the Invention

The present invention relates generally to immunology and genetic engineering technology. Specifically, the present invention relates to immunological engineering to produce novel

reagents that target poisons to cell surface molecules on the cells of microvilli in the midgut of imported fire ant queens.

### Description of the Related Art

5           Imported fire ants are an ecological and financial disaster in Texas as well as other states in the Southern United States. Imported fire ants were accidentally introduced into the U.S. in the 1930s. These pests completely upset and destroy natural ecosystems, and have detrimental economic effects in  
10   agriculture (large mounds damage machinery), ranching (loss of newborn livestock), and recreation and tourism (loss of game birds and rendering park and resort areas uncomfortable at best).

          A specific problem of fire ant control is how one should control or eliminate imported insect species without  
15   destroying native insect species. This problem pertains to numerous non-native animal species that have been introduced in all parts of the United States. Imported species often have a competitive advantage over native species since, in many cases, they have developed enhanced reproductive strategies and do not  
20   have natural predators in their new environment (1). Thus, it is important to eliminate the foreign species. On the other hand, the native species should not be eliminated, as the proper balance of a particular ecosystem includes the presence of that native species.

          Presently, the art includes the various methods of fire  
25   ant control. Chemical poisons, such as AMDRO are well known in the art and are used frequently. Such poisons, however, may pollute the environment, and indiscriminately eliminate native species as well as foreign species.

Thus, the prior art is deficient in an imported fire ant eradication product which is environmentally sound, specifically targeting and eliminating only imported fire ants. The present invention fulfills this long-standing need and desire in the art.

5

## SUMMARY OF THE INVENTION

The present invention is drawn to a safe, cost-effective, environmentally-friendly and ecologically-sound bioengineered product for managing imported fire ants, and a method of making this product. Immunological and genetic engineering techniques are used to generate monoclonal antibodies (mAbs) and resulting Fab fragments as well as viruses (phage) that display antibody fragments, called single heavy or light chain V-gene fragments (scFv) or scFv heavy and light chain fragments (Fab), which exhibit high-avidity specific binding to cells of the microvilli of the midgut of imported fire ant queens. The specific monoclonal antibodies and phage displayed antibody scFv and Fab fragments are conjugated to toxins (gelonin, bacterial endotoxins, or other toxins) or cDNA's coding for pro-apoptotic inducers, cell cycle blockers, cell proliferation inhibitors, differentiation inducers are ligated to scFvFab fragments for targeted delivery and destruction of imported fire ant queens, but not native species, thereby restoring the natural ecosystem. Furthermore, bispecific Fab's or scFv, with one arm of the Fab exhibiting specificity to the targeted cell membrane extracellular domain, and the other arm of the Fab exhibiting specificity to

gelonin, bacterial endotoxin or other toxins provides yet another novel method for specific targeted delivery of toxins. DNA sequence coding regions of the enzymatically active domain of gelonin, bacterial endotoxin or other toxins inserted into DNA coding specific scFv heavy or light chain Ig fragments or Fab Ig fragments provides another method of targeted delivery of toxins.

One object of the present invention is to provide a specific method for management and control of insect pests that is environmentally friendly and does not harm native animal species.

In an embodiment of the present invention, there is provided compositions which specifically deliver pro-apoptotic, cell cycle inhibitors to target cells.

In yet another aspect of the present invention, there is provided a composition to deliver toxins and cell growth inhibitors to target cells.

In yet another aspect of the present invention, there is provided a method for producing reagents that direct poisons to target cells but not to non-target cells, comprising: immunizing an animal with said target cells to produce monoclonal antibodies; harvesting, enriched spleen cells; hybridizing said spleen cells to a myeloma cell fusion partner using polyethylene glycol; selecting of hybridoma cells by growing on HAT media; screening hybridoma supernatants for production of murine antibodies using ELISA technology and for antibody specificity to midgut microvilli antigens using immunohistological techniques; cloning by limiting dilution, screening hybridoma supernatants, expansion of clones and freezing of positive clones; and obtaining a stable, monoclonal

antibody producing hybridomas, produce supernatants or ascites fluid or prepare purified monoclonal antibody.

In yet another aspect of the present invention, there is provided a method of killing a fire ant, comprising the step of  
5 contacting said ant with the polypeptide produced by the method disclosed herein.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments. These  
10 embodiments are given for the purpose of disclosure.

## BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof  
20 which are illustrated in the appended drawings. these drawings form a part of the specification. It is to be noted however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

25 **Figure 1** depicts schematically the methods of the present invention, including immune priming; cDNA preparation; creation of a phage library; phage selection; verification of specific scFvs and Fabs; and testing thereof.

## DETAILED DESCRIPTION OF THE INVENTION

5           As used herein, the term "monoclonal antibody" or "mAb" refers to an antibody comprised of heavy and light polypeptide chains with specificity to target cells and is generated and selected from a cloned antibody producing cell.

10           As used herein, the term "antibody fragment" or "Fab" refers to immunoglobulin based recognition units of minimum size. V-gene segments from immunoglobulin heavy and light chains that exhibit high affinity to target antigens.

15           As used herein, the "scFv" fragment refers to immunoglobulin based recognition unit of minimum size, a single heavy or light or combined heavy and light chain V-gene Ig fragment with high affinity to target cell.

20           As used herein, the term "bispecific antibody" refers to either chemically derived or DNA technology derived Fab or scFv immunoglobulin fragments with specificity to two different antigenic determinants, i.e., one arm of the Ig specificity unit reacting with targeted antigen and the other arm reacting specifically with toxins such as gelonin or bacterial endotoxins.

25           As used herein the term "toxin" refers to any chemical that behaves in a toxic manner in that it kills cells when incorporated into target cells, by being delivered by three distinct mechanisms: chemically linked to targeted Ig fragment, bispecific Fab technology, or by DNA technology providing scFv heavy chain-toxin cytotoxic domain. A representative toxin is gelonin, a well-



known ribosome inactivating protein or recombinant forms thereof.

As used herein, the term "phage display library" refers to repertoire of up to  $2 \times 10^8$  independent clones of immunoglobulin Fab or scFv fragments.

As used herein, the "pro-apoptotic", "cell cycle blockers", "cell proliferation inhibitors" and "cell proliferation agents" refer to cDNA from genes that control cell proliferation cell cycle, cell differentiation, and cell death that are ligated to monoclonal Fab fragments or scFv heavy and/or light Ig fragments for specific delivery to target cell.

As used herein, the term "phage displayed Fab" and "phage displayed scFv" refers to a repertoire of Fab or scFv heavy and/or light chain Ig fragments that are displayed on phage and selected through antigen binding to target cells.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells And Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A vector is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient mammal. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a change in the physiology of a recipient mammal. For example, in the treatment of retroviral infection, a compound which decreases the extent of infection or of physiologic damage due to infection, would be considered therapeutically effective.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells, for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

5           A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms.  
10       Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e.,  
15       the strand having a sequence homologous to the mRNA).

          The present invention is directed to a fire ant eradication product which is environmentally sound, and specifically targets and eliminates only imported fire ants. It is contemplated additionally that the method of the present  
20       invention can be used to specifically target any animal pest.

          Production and screening of monoclonal antibodies with high avidity to specific antigenic epitopes is a well-established and standard laboratory procedure. DNA technology well known to those having ordinary skill in this art permits the  
25       introduction of DNA coding for small immunoglobulin recognition units (called antibody fragments i.e., N-terminal variable domains of heavy and light immunoglobulin chains that exhibit the same antigenic specificity as the intact larger parent antibody) into

virus expression vectors (phage) that produce and display the scFv heavy and light chains and combinations of heavy and light chain Ig fragments on their surface (2-9). This technology has been used to specifically target tumor cells for selected  
5 destruction; however, to date, this technology has not been applied to specifically target, for destruction, insect pests or other animal pests.

The phage display method represents a major advance over traditional monoclonal antibodies in that large and diverse  
10 repertoires of scFv heavy or light and combinations of heavy and light chain Ig v-region genes can be generated and expressed on the surface of viruses; thereby permitting rapid screening and selection for high-avidity (tight binding) scFv Ig fragments with targeted specificity. Importantly, once specific phage-displayed  
15 scFv Ig fragments have been selected for specificity to an antigenic epitope, the DNA that codes for the specific Fab fragment is available for genetically-engineered enzymatically active domain of gelonin, bacterial endotoxins, or other toxins, programmed cell death (apoptotic) genes as well as genes that  
20 disrupt cell proliferation into the Fab DNA. Thus, a targeting system is produced which delivers specific toxins, apoptosis inducing agents or cell proliferation inhibitors, or cell differentiation inducing agents. Producing scFv or Fab Ig fragments with targeted specificity possessing enzymatically  
25 active gelonin or bacterial endotoxins or other cell death inducing gene products provides a novel method for targeted delivery of cell death inducing products.

The present invention utilizes immunological and genetic engineering techniques to generate monoclonal antibodies and viruses (phage) that display antibody scFv and Fab Ig fragments selected to react specifically with the midgut microvilli cells of imported fire ant queens, but not with microvilli cells of native fire ant queens. These Fab and scFv Ig fragments and specificity are not limited to the microvilli or imported fire ant queens, but encompass any cell, tissue or organ specifically targeted with Fab or scFv Ig fragments of any animal species in which the destruction of such cells or tissues or organs will result in the containment or elimination of the animal species. These monoclonal antibodies and phage displayed scFv fragments are conjugated to a toxin, such as gelonin, a ribosome inactivating protein that has no mechanism for entering cells and is non-toxic, unless specifically delivered inside cells, or other toxins, to deliver toxins to the digestive tract of imported fire ant queens.

In yet another aspect of the present invention, there is provided a method of killing a fire ant, comprising the step of contacting said ant with the polypeptide produced by the method disclosed herein. A person having ordinary skill in this art would readily be able to determine the optimum concentration of the novel polypeptides disclosed herein by routine experimentation according to the type and quantity of fire ant to be irradiated.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

### EXAMPLE 1

#### Immunization of mice with midgut microvilli cells

Balb/c mice are immunized with midgut preparations obtained from egg laying imported fire ant queens. Antigenically complex minced midguts are used for immunizations, and then a dual selection procedure (see Example 4) and an immunohistochemical procedure (see Example 5) is performed for selecting either scFv heavy and/or light chain Ig fragments or monoclonal Fab fragments with specificity to cells of the microvilli of the imported fire ant queens.

Spleen cells from immunized mice are used to generate species-specific monoclonal antibodies, using standard cell culture procedures. The monoclonal antibodies have specificity to midgut microvilli antigens of imported fire ants. Resulting hybridoma supernatants are selected for specificity using immunohistochemical procedures as described in Example 5.

In addition, species-specific antibody phage displayed scFv heavy and/or light chain Ig fragments are generated which react with the extracellular domain of midgut microvilli cells. A schematic of the step-by-step procedure in the development of phage displayed scFv heavy and/or light chain Ig fragments is presented in Figure 1.

### EXAMPLE 2

#### Preparation, Amplification and Purification of cDNA from total RNA obtained from immunized mice spleens

Total RNA is extracted and purified from the spleens of three immune mice. The RNA is reverse transcribed (RT) into

cDNA employing a kit (Boehringer Mannheim) and using two sets of primers--one specific for IgG heavy chains and another specific for kappa light chains (4). cDNAs are amplified by the polymerase chain reaction (PCR) procedure using IgG heavy and kappa light chain specific primers, respectively, and thermal cycling conditions published previously (see 4,5,6). The PCR-amplified products are separated according to size by gel electrophoresis.

### EXAMPLE 3

#### Construction of a scFv heavy and/or light chain Ig fragment combinatorial library displayed on the surface of filamentous phage

Combinatorial scFv heavy and light chain Ig fragment libraries are constructed in a filamentous phage expression vector using a two-step sequential ligation procedure as described (4) and an ImmunoZAP kit from Stratagene. Briefly, the gel-purified PCR products are enzymatically cleaved and the PCR products are ligated into the heavy chain vector - IHc2, and the light chain vector-ILc. Constructed heavy and light chain libraries consist of scFv fragments. Next, heavy and light chain constructs are combined to yield Fab combinatorial constructs. *E. coli* cells are then transformed with the phage library and infected with helper phage (VCSM13, Stratagene) to produce sufficient amounts of phage displaying antibody fragments for screening and selection purposes (4). scFv heavy and light as well as heavy and light chain Ig V-region specificity fragments from immunoglobulin heavy and light chain are utilized as either single chain V-

fragments (scFv) of high binding affinity to target cells or as a combined heavy/light chain V-fragments (Fab) with high binding affinity to target cells.

5

#### EXAMPLE 4

Two-step selection for identifying antibody scFv or Fab fragments with specificity to midgut membrane antigens from imported fire ant queens

10           The first selection step involves reacting the entire antibody scFv or Fab fragment-expressing phage library with midguts opened to expose microvilli cells or cells dissociated, obtained from imported fire ant queens. Ig displayed phage failing to bind are washed away while phage displaying scFv or  
15 Fab Ig fragments that bind to imported fire ant midgut antigens with high affinity are eluted and saved. The second selection is conducted using midgut preparations from native fire ant queens. Only phage-displayed scFv or Fab fragments that fail to react with midgut membrane antigens from native fire ant queens are  
20 collected. This selection procedure is repeated three times to provide specific Ig fragments with high avidity and affinity for cloning. The selected phage (those that react with midgut membrane antigens of imported fire ant queens but not native fire ant queens) are genetically engineered so that they no longer  
25 are displayed, but are secreted when produced in E. coli. The selection procedure is not limited to the above sequence, in that, Ig displayed phage can be first reacted with microvilli antigens of native fire ant queens, and Ig displayed phage not reacting with



the native fire ant queen microvilli antigens can be selected for reaction with microvilli antigens of imported fire ant queens.

### EXAMPLE 5

5

Verification that selected phage displayed antibody scFv or Fab fragments and monoclonal antibodies react with imported fire ant midgut microvilli cells

The selected phage-displayed scFv or Fab fragments  
10 (from Example 4, above) react with a variety of midgut cellular membrane antigens, including antigens of the microvilli cells. Immunohistochemical staining of sectioned midgut tissue is used to identify cloned phage-displayed scFv heavy and light chains or combinations of heavy and light chain Ig fragments that react  
15 with midgut microvilli cells of imported fire ant queens exclusively. Midgut sectioned tissue is reacted with cloned scFv or Fab fractions, and assayed for the presence of the phage using the sensitive immunoperoxidase VECTASTAIN Elite ABC system (Vector Laboratories). Clones that give a positive reaction with  
20 microvilli cells of imported fire ant queens and fail to react with native fire ant microvilli cells are amplified in E. coli and used as described in Example 6 and 7. For selection of monoclonal antibodies, midgut tissue sections are reacted with hybridoma supernatants, washed, and reacted with alkaline phosphatase-  
25 conjugated rabbit secondary antibody to mouse Ig. Substrate is then added and slides are examined for positive reactions to the midgut microvilli antigens of imported fire ant queens but not the midgut microvilli antigens of native fire ant queens.

**EXAMPLE 6**

Verification that the selected phage-displayed antibody fragments, as well as monoclonal antibodies, are effective in vivo

5            Monoclonal antibodies and scFv heavy and/or light chain Ig fragments amplified in *E. coli* that are selected for specificity and high affinity binding to the microvilli of imported fire ants are tested for ability to be internalized by microvilli cells when administered orally. Ant colonies (imported and native) are  
10   established in a controlled environment with each colony consisting of a single egg laying imported fire ant queen, 50 virgin winged fire ant Queens and approximately 5,000 worker ants. The colonies are fed either monoclonal antibodies or scFv or Fab fragments mixed in soybean meal. The egg laying queen and  
15   virgin queens from each colony are sacrificed and the midguts are removed. Frozen tissue sections are made from the midgut, and these sections are analyzed for the presence and internalization of scFv heavy and/or light chain Ig fragments by immunohistochemical staining as described above. Analyses of  
20   monoclonal Fab fragments are conducted using this methodology.

**EXAMPLE 7**

Verification that phage-displayed generated scFv Ig fragments and monoclonal Fab fragments are an effective delivery system for targeted destruction of imported fire ants:

25

The ribosome inactivating protein, gelonin, bacterial endotoxins, or other toxins are attached to monoclonal Fab

fragments and scFv heavy and/or light chain Ig fragments by the following technologies: by well established chemical conjugation or attachment procedures that have been successful in conjugation of toxins to monoclonal antibody Fab fragments or to scFv heavy chain Ig fragments; by bispecific (Fab)<sub>2</sub> or scFv heavy chain heterodimers with specificity to targeted antigens and specificity to toxin generated in vitro by establishing a stable thioether linkage using established procedures (10-12); in vivo by using DNA technology and genetic engineering technologies to generate dimerization peptides to produce bivalent dimers (with specificities to targeted antigen and toxin) in E coli or mammalian cells, using described techniques (13), and by genetically engineering scFv heavy chain-enzymatically active domain of toxin, expressed and secreted by bacteria using described technology (14).

The testing "feeding" of scFv heavy and/or light chain or monoclonal Fab fragments for ability to bind to midgut microvilli antigens of imported fire ant queens but not midgut microvilli antigens of native fire ant queens in controlled established laboratory colonies of imported and native fire ants, utilizing immunohistological techniques to establish attachment and internalization of Ig fragments to midgut microvilli antigens is performed. Testing of targeted scFv heavy and/or light chains and monoclonal Fab fragments + toxin in laboratory established ant colonies as well as in field conditions for ability to selectively kill imported fire ants is performed.

The following references were cited herein:

1. Holldobler, B. and E. O. Wilson 1990. The Ants. The Belknap Press, Cambridge, Mass.
2. Barbas, C. and R. Lerner. 1991. Combinatorial immunoglobulin libraries on the surface of phage (phabs): rapid selection of  
5 antigen-specific. *Fab. Methods: Comp. Met. Enzym.* 2:119.
3. Ames, R., M. Tornetta, C. Jones and P. Tsui. 1994. Isolation of neutralizing anti-C5a antibodies from a filamentous phage monovalent Fab display library. *J. Immun.* 152:4572.
4. Ames, et al (15 co-authors). 1995. Neutralizing murine  
10 monoclonal antibodies to human IL-5 isolated from hybridomas and a filamentous phage Fab display library. *J. Immunol.* 154:6355.
5. Winter, G., A. D. Griffiths, R. E. Hawkins and H. R. Hoogenboom. 1994. Making antibodies by phage display technology. *Ann. Rev.*  
15 *Immunol.* 12:433.
6. Vaughan, T. J., et al., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotech.* 14: 309.
7. Kruif, J. de, et al., 1996. New perspectives on recombinant  
20 human antibodies. *Immunology Today* 17:453.
8. Kruif, J. de, and T. Logtenberg. 1996. Leucine zipper dimerized bivalent and bispecific scFv antibodies from a semi-synthetic antibody phage display library. *J. Bio. Chem.* 271:7630.
9. Davies, J. and L. Riechmann. 1995. Antibody VH domains as  
25 small recognition units. *Biotechnology* 13:475.
10. French, R, C. Penney, A. Browning, F. Stirpe, A. George and M. Glennie. 1995. Delivery of the ribosome-inactivating protein,

gelonin, to lymphoma cells via CD22 and CD38 using bispecific antibodies. Brit. J. Cancer 71:986.

11. Better, M., S. Bernhard, D. Fishwild, P. Nolan, R. Bauer, A. Kung, and S. Carroll. 1994. Gelonin analogs with engineered cysteine residues form antibody immunoconjugates with unique properties. J. Biol. Chem. 269:9644.
12. Glennie, M.J., H. M. McBride, A. T. Worth, and G. T. Stevenson. Preparation and performance of bispecific F(ab')<sub>2</sub> antibody containing thioether-linked Fab' fragments. J. Immunol. 139:2367-2375, 1987.
13. Holliger, P., and G. Winter. Engineering bispecific antibodies. Current Opinion in Biotechnology 4: 446-449, 1993.
14. Maurer-Gebhard, M., M. Schmidt, M. Azemar, U. Altenschmidt, E. Stocklin, W. Wels, and B. Groner. Systemic treatment with a recombinant erbB-2 receptor-specific tumor toxin efficiently reduces pulmonary metastases in mice injected with genetically modified carcinoma cells. Cancer Res. 58:2661-2666, 1998.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments,

molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which  
5 are encompassed within the spirit of the invention as defined by the scope of the claims.

**WHAT IS CLAIMED IS:**

1. A method of producing a reagent with specificity  
5 to a target antigen and an active domain of a toxin, comprising the steps of:
  - creating an scFv heavy and/or light chain Ig fragment-expressing phage display library;
  - reacting said scFv heavy and/or light chain Ig fragment-  
10 expressing phage display library with said target cells;
  - washing said reacted target cells to remove unbound scFv Ig fragment-expressing phage;
  - eluting bound scFv Ig fragment-expressing phage from said target cells;
  - 15 reacting said eluted scFv Ig fragment-expressing phage with said non-target cells;
  - washing said reacted non-target cells to remove unbound, eluted scFv Ig fragment-expressing phage;
  - engineering scFv for non-phage display;
  - 20 amplifying scFv heavy and/or light chain Ig fragments in *E. coli*;
  - collecting secreted scFv Ig fragments;
  - attaching said scFv Ig fragments to a toxin to produce scFv heavy chain-enzymatically active domain of toxin thus producing  
25 a single polypeptide with specificity to targeted antigen and active domain of toxin.

2. The method of claim 1, wherein said attaching is by chemical cross-linking, bispecific scFv heavy and light chain, with specificity to targeted antigen and toxin, or genetic engineering.

5

3. The method of claim 1, wherein said animal is a mouse.

4. The method of claim 1, wherein said target is a  
10 cell surface molecule of a target cell.

5. The method of claim 1, wherein said target cell is a cell in the microvilli of the midgut region of an imported fire ant.

15 6. The method of claim 1, wherein said creating step is carried out by ligating cDNA into heavy chain vectors and light chain vectors.

7. A polypeptide produced by the method of claim  
20 1.

8. A method for producing monoclonal antibody and Fab fragments with specificity to midgut microvilli antigens of imported fire ant queens, comprising:

25 immunizing an animal with said target cells to produce monoclonal antibodies;

harvesting enriched spleen cells;



hybridizing said spleen cells to a myeloma cell fusion partner using polyethylene glycol;

selecting of hybridoma cells by growing on HAT media;

screening hybridoma supernatants for production of  
5 murine antibodies using ELISA technology and for antibody specificity to midgut microvilli antigens of imported fire ant queens but not midgut antigens of native fire ant queens using immunohistological techniques;

cloning by limiting dilution, screening hybridoma  
10 supernatants, expansion of clones and freezing of positive clones; and

purifying monoclonal antibody and Fab fragments with specificity to midgut microvilli antigens of imported fire ant queens.

15

9. The method of claim 8, wherein said animal is a mouse.

10. The method of claim 8, wherein said target cell is  
20 a cell in the microvilli of the midgut region of an imported fire ant.

11. A monoclonal antibody or Fab fragment produced by the method of claim 8.

25

12. A method of killing a fire ant, comprising the step of contacting said ant with the polypeptide produced by the method of claim 1.

**IMMUNE PRIMING**

Immunize mice with imported  
fire ant midgut tissue

**RNA → cDNA**

Isolate total RNA from spleen of immunized mice,  
prepare cDNA by reverse transcription, amplify  
by polymerase chain reaction, purify cDNA from gel

**ANTIBODY LIBRARY ON  
SURFACE OF PHAGE**

Create phage display library  
expressing  $10^6$ - $10^8$  unique  
antibody Fab fragments

**DUAL MIDGUT SELECTION**

Two-step absorptions to yield  
phage displaying antibody fragments  
specific for midgut of imported fire ants  
and not native fire ants

**FINAL MICROVILLI SELECTION**

Immunohistochemical verification of  
Fab specific to imported fire ant  
microvilli cells

**TESTING**

Test phage/Fab for internalization by  
microvilli cells of imported fire ants  
when administered by feeding

**IMPORTED FIRE ANT ERADICATION**

Test phage/Fab/gelonin Conjugate for ability  
to selectively kill imported fire ants.

FIGURE 1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/14216

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : G01N 33/53; C07K 16/00; A61K 39/395

US CL :435/7.1; 530/387.1; 424/130.1, 178.1, 236.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1; 530/387.1; 424/130.1, 178.1, 236.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG. search terms: scfv, single chain antibody, phage display, immunotoxin, midgut microvilli, imported fire ant queen,

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUSTON et al. Medical applications of single chain antibodies. Intern. Rev. Immunol. 1993, Vol. 10, pages 195-217, see entire document.	1-4, 6-7
X	LORIMER et al. Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: Targeting with a single chain antibody variable domain isolated by phage display. Proc. Natl. Acad. Sci. USA. December 1996, Vol. 93, pages 14815-14820. See entire document.	1-4, 6-7

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

## \* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*Z\* document member of the same patent family

Date of the actual completion of the international search

21 SEPTEMBER 1998

Date of mailing of the international search report

2.2 OCT 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MINH-TAM DAVIS

Telephone No. (703) 308-0916

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/14216

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/14216

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-7, drawn to a method for producing a single chain immunotoxin by phage display, and a polypeptide produced by said method.

Group II, claim(s) 8-10, drawn to a method for producing antibody to midgut microvilli of imported fire ant queen.

Group III, claim(s) 11, drawn to a monoclonal antibody or Fab fragment.

Group IV, claim(s) 12, drawn to a method for killing fire ant.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

An international stage application shall relate to one invention only or to a group of invention so linked as to form a single general inventive concept. If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application will be considered as the main invention in the claims, see PCT article 17(3) (a) and 1.476 (c), 37 C.F.R. 1.475(d). Group I will be the main invention. After that, all other products and methods will be broken out as separate groups (see 37 CFR 1.475 (d)).

Group I, claims 1-7 form a single inventive concept, drawn to a method of producing single chain immunotoxin, and a single chain immunotoxin. Group II is an additional method, i.e a method for producing antibodies. Group III is an additional product, an antibody specific for midgut microvilli of fire ant queen, and group IV is an additional method, i.e. a method for killing fire ant, using said single chain immunotoxin.